Effect of iron concentration on the expression and activity of catalase-peroxidases in mycobacteria

Veena C Yeruva, C A S Sivagami Sundaram and Manjula Sritaran*
Department of Animal Sciences, School of Life Sciences, University of Hyderabad,
Hyderabad 500 046, India

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Mycobacterial catalases are known to exist in different isoforms. We studied the influence of iron concentration on the expression and activity of the different isoforms in Mycobacterium bovis BCG, M. smegmatis, M. fortuitum, M. kansasii and M. vaccae by growing them under iron-sufficient (4 µg Fe/mL) and iron-deficient (0.02 µg Fe/ml) conditions. Upon iron deprivation, significant differences were observed in the catalase/peroxidase activities in both quantitative spectrophotometric assays and in the activity staining in native gels. Notable feature was that the peroxidase activity showed a significant decrease upon iron deprivation in all the mycobacteria, except M. vaccae. Peroxidase activity in all the mycobacteria, irrespective of the iron status was susceptible to heat inactivation. However, the isoforms of catalase showed differences in their heat stability, indicating possible structural differences in these proteins. For example, M. bovis BCG expressed a heat labile catalase under iron-sufficient conditions, while a heat stable catalase band of similar mobility was expressed under iron-deprivation conditions. The study clearly indicates that iron plays an important role in the regulation of expression of the different isoforms of the catalase-peroxidases.

Keywords: catalase-peroxidases, mycobacteria, iron.

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Microorganisms though face conditions of iron deprivation, as Fe(III) exists either as insoluble Fe(OH)₃ at neutral pH or as protein-bound iron in the mammalian system, held by transferrin, lactoferrin and ferritin, they adapt to such conditions by elaborating unique mechanisms of iron acquisition. Mycobacteria also adapt to the iron-deficient condition and express siderophores (intracellular mycobactin and extracellular exochelin) and iron-regulated envelope proteins (IREPs). Interestingly, IREPs have also been demonstrated in the in vivo derived mycobacteria. The co-ordinated expression of the siderophores and IREPs was demonstrated in Mycobacterium neoaurum. At molecular level, IdeR iron regulator protein controls the expression of the components of iron acquisition machinery. The iron regulator Fur, first identified in gram-negative organisms is also found in mycobacteria. Two Fur-like proteins that are known to regulate several genes involved in iron metabolism have been identified in the genome of M. tuberculosis. The furA gene, coding for one of the two Fur-like proteins is located upstream of the katG gene, coding for catalase-peroxidase and the fur-katG organization is conserved in several mycobacteria.

Catalases and peroxidases are oxido-reductases involved in the molecular mechanisms of defense against reactive oxygen species. Although they possess striking similarities in the reaction mechanism, they have different residues involved in the haeme cavity. The enzymes that exhibit either catalatic or peroxidative activity are common among animals, plants and microorganisms. Prokaryotes express catalase-peroxidases possessing both the activities in a single protein molecule, suggesting the common phylogeny of the catalase and peroxidative activities during evolution. In mycobacteria, two classes of catalases, namely the T and M are recognised. The T class is the heat labile enzyme with
peroxidase activity and the M class is the heat stable form with catalase activity*. Multiple catalases have been demonstrated in mycobacteria*.11

Earlier catalase1,12 and iron concentrations13,14 have been implicated in the virulence of mycobacteria. Here, we have attempted to investigate the interrelationship between these components and studied the effect of iron concentration on the peroxidase and catalase activities in different mycobacteria.

Materials and Methods

Mycobacterial strains

*Mycobacterium smegmatis* mc²155, *M. kansasii* (ATCC 12478), *M. bovis* BCG Danish (kindly given by Dr. Peter Small, Stanford University, USA), *M. vaccae* and *M. fortuitum* (National Repository for Mycobacterial Cultures at Central JALMA Institute for Leprosy, Agra, India) were used in the study.

Growth of mycobacteria under iron-sufficient and iron-deficient conditions

The different mycobacterial strains were grown in modified Proskauer and Beck medium² under iron-sufficient (4 μg Fe/ml) and iron-deficient (0.02 μg Fe/ml) conditions.

Assay for the expression of siderophores

The establishment of iron-deficient conditions was assayed by the universal chrome azurol S (CAS) assay15. Briefly, 1 mL of culture filtrate was added to 2 mL of CAS solution and the absorbance at 630 nm was measured after 30 min incubation at room temperature. The concentration of the siderophore is expressed as siderophore units. One siderophore unit= (A_C-A_s/A_C) × 100, where A_C represents the absorbance of CAS solution plus medium and A_s, the absorbance of the CAS solution plus culture filtrate of the respective sample.

Preparation of cell-free cell extracts

The cells, washed and re-suspended in phosphate buffer (0.05 M, pH 7.0) were sonicated in a Vibra cell sonicator (12 pulses with each pulse for 20 sec at 12 Hz). The sonicate was centrifuged at 8000xg for 20 min to obtain the cell-free extract, which was stored at -20°C. Protein concentration was estimated by the method of Lowry et al.16

Enzyme assays

Catalase activity was determined spectrophotometrically17 by measuring the decrease in H₂O₂ concentration by reading the absorbance at 240 nm (E₂₄₀ = 0.0435 mM⁻¹ cm⁻¹) and the specific activity of catalase was expressed as micromoles of H₂O₂ decomposed per min per mg of total protein. Peroxidase activity was assayed in a microtitre plate by incubating with the tetramethyl benzidine (TMBZ) substrate in citrate buffer at pH 5.0 (ready-to-use solution purchased from Bangalore Genei, India). The activity was expressed as OD₆₃₀ nm units per mg of total protein.

Activity staining for catalase in non-denaturing native gels

Native PAGE of the cell-free extracts was performed under non-denaturing conditions. The bifunctional catalase-peroxidase activity was detected by the double staining method³.

Results

Establishment of iron deficient conditions: CAS assay

Iron-deficiently grown (0.02 μg Fe/ml) *M. kansasii, M. fortuitum, M. smegmatis* and *M. bovis* BCG expressed significant levels of the siderophore as compared to iron-sufficient cells (4 μg Fe/ml) and in *M. vaccae*, no appreciable difference was noted (Fig. 1).

Catalase-peroxidase activities in iron-sufficient and iron-deficient mycobacteria

Iron deprivation resulted in a 3-5-fold decrease in the catalase activity in *M. fortuitum* and *M. kansasii* and a 2-fold decrease in *M. bovis* BCG and *M. smegmatis*. Under iron-sufficient conditions,
Fig. 2—Spectrophotometric assay of catalase (A) and peroxidase (B) in iron-sufficient and iron-deficient cells of *M. bovis* BCG, *M. fortuitum*, *M. kansasii*, *M. smegmatis* and *M. vaccae* [The catalase activity was measured by the decrease in the H$_2$O$_2$ concentration and the peroxidase by reactivity with the TMBZ substrate as described in Materials and Methods. The vertical bars represent the standard deviation of the mean from 3 independent experiments]

*M. kansasii* expressed highest level of catalase activity and *M. smegmatis* the least. The notable exception was *M. vaccae* which showed no significant change in the catalase activity upon iron deprivation (Fig. 2A).

Peroxidase activity of *M. bovis* BCG, *M. fortuitum* and *M. kansasii* was significantly decreased (10-13-fold) upon iron deprivation, while a smaller decrease was observed in *M. smegmatis*. *M. vaccae* showed no change in the activity with reference to the iron concentration in the culture medium (Fig. 2B). Even under iron-sufficient conditions, the peroxidase activity of *M. smegmatis* and *M. vaccae* was much lower than other mycobacteria.

Activity staining of catalase-peroxidase in native gels

The effect of iron concentration on the expression of the different isoforms of catalase-peroxidase and their susceptibility to heat treatment was visualized in native gels by activity staining (Fig. 3). The different bands reflect only the mobility of the enzyme in the gel and do not indicate whether the enzymes with similar gel mobility are identical. Hence, the bands are considered as distinct proteins. The different isoforms of the catalase-peroxidase show variation, with bands of either catalase or peroxidase activity or both as seen in *M. bovis* BCG (Fig. 3A), *M. smegmatis* (B), *M. vaccae* (C) and *M. fortuitum* (D) and lanes 3 and 4, the activity of identical extracts subjected to heat treatment at 55°C for 10 min.

The progressive inactivation of the peroxidase and catalase activities with increasing temperature was seen in all mycobacteria tested and the observations in *M. kansasii* are noteworthy. The observations in the different mycobacteria are summarized in Table 1.

The progressive inactivation of the peroxidase and catalase activities with increasing temperature was seen in all mycobacteria tested and the observations in *M. kansasii* are presented. The quantitative decrease in the peroxidase (Fig. 4A) and catalase activities
Table 1—Characteristics of the multiple catalase-peroxidases of mycobacteria grown under iron-sufficient (4 µg Fe/ml) and iron-deficient (0.02 µg Fe/ml) conditions

<table>
<thead>
<tr>
<th>Species</th>
<th>Iron status*</th>
<th>Isoforms</th>
<th>Enzyme activity</th>
<th>Characteristics of catalase/peroxidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. bovis BCG</td>
<td>2</td>
<td>Dual</td>
<td>Significant peroxidase activity in both isoforms. Both peroxidase and catalase</td>
<td>Heat labile</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Catalase</td>
<td>Complete loss of peroxidase; one significant heat stable catalase</td>
<td></td>
</tr>
<tr>
<td>M. fortuitum</td>
<td>4</td>
<td>Dual-1</td>
<td>Low level of peroxidase; both peroxidase and catalase are heat labile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Peroxidase-2</td>
<td>Significant peroxidase; heat labile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Catalase-1</td>
<td>Significant catalase; heat labile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Peroxidase-2</td>
<td>Low levels of activity; heat labile</td>
<td></td>
</tr>
<tr>
<td>M. kansasii</td>
<td>4</td>
<td>Dual-3</td>
<td>Significantly high peroxidase and catalase activity; both were heat labile</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Catalase-1</td>
<td>Complete loss of peroxidase; one significant heat stable catalase</td>
<td></td>
</tr>
<tr>
<td>M. vaccae</td>
<td>2</td>
<td>Catalase-2</td>
<td>One major band of heat stable catalase activity. Another band showed weak activity.</td>
<td></td>
</tr>
<tr>
<td>M. smegmatis</td>
<td>4</td>
<td>Catalase-1</td>
<td>Heat stable catalase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Dual-3</td>
<td>Heat labile peroxidase, with one band of heat resistant catalase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Dual-2</td>
<td>Heat labile peroxidase and catalase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Catalase-1</td>
<td>Prominent band of heat stable catalase</td>
<td></td>
</tr>
<tr>
<td>M. smegmatis</td>
<td>1</td>
<td>Catalase-1</td>
<td>Heat stable catalase</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Peroxidase-3</td>
<td>One prominent band, with two faint bands. All were heat labile.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Dual-1</td>
<td>Significant catalase with weak peroxidase activity, both of which were unaffected by heat</td>
<td></td>
</tr>
</tbody>
</table>

*The arrows ↑ and ↓ indicate iron-sufficient and iron-deficient conditions of growth

Fig. 4—Effect of increasing temperature on the catalase and peroxidase activities of iron-sufficient and iron-deficient cells of M. kansasii

[The peroxidase (A) and catalase (B) activities were assayed both spectrophotometrically as described in 'Materials and Methods']
Fig. 4C—Activity staining of native gels showing the effect of temp. on catalase and peroxidase activities of iron-sufficient and iron-deficient cells of M. kansasii [The lanes 1, 3, 5, 7 and 9 represent the cell-free extracts of iron-sufficient cells and lanes 2, 4, 6, 8 and 10 represent iron-deficient cells incubated for 30 min at temp. (°C) of 37, 40, 45, 50 and 60 respectively]

(Fig. 4B) with increasing temperature were also reflected in the activity staining of identical samples (Fig. 4C). The differences in the susceptibilities of different isoforms to heat were evident from the activity staining.

Discussion
In this study, conditions of iron deprivation were successfully established for M. fortuitum, M. kansasii, M. smegmatis and M. vaccae and for the slow growing M. bovis BCG, as assessed by the CAS assay. The most notable observation was the sharp decrease in the peroxidase activity upon iron deprivation; the effect was highly pronounced in M. kansasii, M. fortuitum and M. bovis BCG. The distribution of high levels of peroxidase activity among the various isoforms in iron-sufficient cells was variable in the different mycobacteria. The peroxidase activity, irrespective of the iron status was heat labile in the mycobacteria tested. In M. vaccae, though no significant differences in the catalase and peroxidase activities were observed spectrophotometrically between iron-sufficient and iron-deficient cells, qualitative differences could be seen upon activity staining.

Interestingly, under conditions of iron deprivation, the catalase activity was not affected to a great extent. Contrary to the general notion that all catalases are heat stable, we have observed that the catalases expressed by iron-deficient cells were more heat stable, as compared to the iron-sufficient cells. For example, in M. bovis BCG, the catalase expressed by iron-sufficient cells was heat labile, while under iron-deficient conditions, the catalase band showing similar mobility was heat stable. It is likely that structural differences contribute to their susceptibility to heat. Whether this alteration in the enzyme activities confers an advantage to the organism in environments of varying iron concentration needs to be studied further.

Thus, it can be inferred from the above observations that in mycobacteria used in the study, the expression of different isoforms of the catalase-peroxidase with alterations in the enzyme activities is regulated at the molecular level by iron. It is reported earlier that the FurA protein controls the expression of the katG gene. Recently, it has been shown that the regulation of the katG gene by the FurA protein is much more complex, as two more regions pfurA and pkatG were identified in the control of expression of the katG gene. Although the exact mode of the FurA action is not yet clear, the control of catalase-peroxidase expression at the post-transcriptional level by the FurA was considered as likely. It is now clear in many bacteria that iron acts as a regulator; upon binding to Fur and Fur-like repressor, it controls the expression of both the components of the iron-acquisition machinery and bacterial toxins. The present study shows the direct effect of iron concentration on the catalase and peroxidase activities in mycobacteria, the implications of which need to be studied further.

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